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Ancient Heavy Metal Contamination in Soils as a Driver of Tolerant *Anthyllis vulneraria* Rhizobial Communities

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ABSTRACT *Anthyllis vulneraria* is a legume associated with nitrogen-fixing rhizobia that together offer an adapted biological material for mine-soil phytostabilization by limiting metal pollution. To find rhizobia associated with *Anthyllis* at a given site, we evaluated the genetic and phenotypic properties of a collection of 137 rhizobia recovered from soils presenting contrasting metal levels. Zn-Pb mine soils largely contained metal-tolerant rhizobia belonging to *Mesorhizobium metallidurans* or to another sister metal-tolerant species. All of the metal-tolerant isolates harbored the *cadA* marker gene (encoding a metal-efflux P_{IB}-type ATPase transporter). In contrast, metal-sensitive strains were taxonomically distinct from metal-tolerant populations and consisted of new *Mesorhizobium* genospecies. Based on the symbiotic *nodA* marker, the populations comprise two symbiovar assemblages (potentially related to *Anthyllis* or *Lotus* host preferences) according to soil geographic locations but independently of metal content. Multivariate analysis showed that soil Pb and Cd concentrations differentially impacted the rhizobial communities and that a rhizobial community found in one geographically distant site was highly divergent from the others. In conclusion, heavy metal levels in soils drive the taxonomic composition of *Anthyllis*-associated rhizobial populations according to their metal-tolerance phenotype but not their symbiotic *nodA* diversity. In addition to heavy metals, local soil physicochemical and topoclimatic conditions also impact the rhizobial beta diversity. *Mesorhizobium* communities were locally adapted and site specific, and their use is recommended for the success of phytostabilization strategies based on *Mesorhizobium*-legume vegetation.

IMPORTANCE Phytostabilization of toxic mine spoils limits heavy metal dispersion and environmental pollution by establishing a sustainable plant cover. This eco-friendly method is facilitated by the use of selected and adapted cover crop legumes living in symbiosis with rhizobia that can stimulate plant growth naturally through biological nitrogen fixation. We studied microsymbiont partners of a metal-tolerant legume, *Anthyllis vulneraria*, which is tolerant to very highly metal-polluted soils in mining and nonmining sites. Site-specific rhizobial communities were linked to taxonomic composition and metal tolerance capacity. The rhizobial species *Mesorhizobium metallidurans* was dominant in all Zn-Pb mines but one. It was not detected in unpolluted sites where other distinct *Mesorhizobium* species occur. Given the different soil conditions at the respective mining sites, including their heavy-metal contamination, revegetation strategies based on rhizobia adapting to local conditions are more likely to succeed over the long term compared to strategies based on introducing less-well-adapted strains.

KEYWORDS symbiotic nitrogen fixation, metal tolerance, multilocus sequence analysis, nodulation gene, P_{IB}-type ATPase, *Aminobacter*, *Mesorhizobium*, phytostabilization

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Metals released by anthropogenic activities, such as from metalliferous mines, are a major cause of environmental pollution. Such mining environments are highly vulnerable to soil erosion that results in extensive air and water pollution (1). As metals cannot be degraded or destroyed, they persist within natural environments and disturb ecosystem dynamics. As a consequence, they pose a significant threat to public health (2) and to natural environments (3, 4). To limit metal pollution, it is necessary to rehabilitate metal-contaminated soils as thoroughly as possible. Among the phytoremediation strategies, vegetation and phytostabilization offer environmentally friendly and cost-effective solutions for limiting the impact of metals on the environment (5, 6). The practical goal is to develop a sustainable solution based on microbe-assisted phytoremediation that will restore the vital functions of soil and plant ecosystems and sequester the metals in root systems to prevent further spreading and leaching of the metal into soil and ground water. In most cases, this strategy relies on establishing plants adapted to metal toxicity and to reduced water and nutrient availability (7). Heavy metal-contaminated soils, notably mine tailings, are severely depleted of organic materials, particularly nitrogen (8). Consequently, in such phytomanagement, it is pivotal to include biological nitrogen-fixing plants, such as those from the Fabaceae family, which can efficiently interact with symbiotic bacteria (rhizobia), for natural soil nitrogen enrichment that improves soil properties and plant growth and succession (9, 10).

Rhizobia are important drivers of environmental and agricultural services and the impact from heavy metals has been extensively studied. Elevated metal concentrations influence microbial populations by affecting their growth, abundance, diversity (11–13), and activity (14). Metals exert selection pressure on microorganisms, resulting in the emergence of microbial populations with a higher tolerance to metals but with a lower diversity compared to that of unpolluted neighboring areas (11, 15, 16). Such selection pressures may also lead to drastic changes in rhizobial populations (17). The metal tolerance can be conferred by the acquisition of plasmids or genomic islands (18, 19) harboring metal-resistance genes encoding metal efflux systems or genes involved in intra- or extracellular metal sequestration, precipitation, and reduction to less toxic forms (20). In a given environment, the ratio of metal-resistant to -sensitive microorganisms may be seen as a bioindicator of the degree of pollution (21, 22). However, some reports found that with long-term contamination, metals such as zinc (Zn) and cadmium (Cd) did not decrease rhizobial diversity (23) or rhizobial numbers (24). The effects that metals have on microbial diversity and composition (25) depend upon the metal availability, which is influenced by other factors such as climate (26), soil type and structure (27, 28), presence of organic matter (29), pH (30), and plant roots (31).

In the mining area of Saint-Laurent-le-Minier (France), which represents one of the most polluted sites in Europe due to high levels of Zn, Cd, and lead (Pb), the association between a legume species, *Anthyllis vulneraria*, and Poaceae plants (*Festuca*, *Koeleria* spp.) successfully restored a sustainable vegetation cover, thereby limiting the impact of metals on the environment (32). The *A. vulneraria* ecotype from the Saint-Laurent mine belongs to the subspecies *carpatica*. Rather than a hyperaccumulator, this species is considered a metal-tolerant accumulator able to grow in contaminated soil (33) and which tolerates high Zn concentrations unlike non-metal-tolerant *Anthyllis* subspecies (34). The rhizobial symbionts that associate with subspecies *carpatica* belong to the species of *Mesorhizobium metallidurans* (35) that efficiently nodulate and fix nitrogen at rates that range from 70 to 80% (36). Besides being an efficient symbiont of *Anthyllis*, *M. metallidurans* is resistant to Zn and Cd. Metal-tolerant bacteria display widely represented mechanisms of metal tolerance, and several genes encoding metal efflux and sequestration systems significantly upregulate in response to sublethal metal doses in the type strain of *M. metallidurans* (37, 38). Such mechanisms may confer a selective advantage to *M. metallidurans* populations in highly contaminated soils.

M. metallidurans was first isolated in the Saint-Laurent mine (Avinières site) and, until now, had not been reported from other places, raising the question about its occurrence in other soils. Given the potentially positive impact of the *M. metallidurans*-*Anthyllis*-mediated phytostabilization strategy observed at the Saint-Laurent mine site,

a better knowledge of the natural *Anthyllis* rhizobial diversity in relation to metal content may facilitate the detection and selection of appropriate symbiotic bacteria to be used in other Zn-, Cd-, and/or Pb-contaminated sites needing restoration. This study explored the ecology and the geographic distribution of metal-tolerant symbiotic bacteria nodulating *Anthyllis* from soils originating from four mines (M1 to M4) across western Europe, as well as two moderately contaminated soils from mine borders (B1 and B2) and two unpolluted nonmining sites (NM1 and NM2). We analyzed taxonomic, symbiotic, and metal-resistance markers, which, combined with phenotypic tests, enabled us to estimate the taxonomic and functional diversity of *Anthyllis*-nodulating rhizobia in relation to the contamination levels of the sampled soils.

RESULTS

Soil characteristics. The edaphic properties varied in the eight sampled sites (see Table S3 in the supplemental material). The four mine soils (M1 to M4) contained the highest EDTA-extractable metal concentrations (15.47 to 40.2 g Zn kg^{-1}, 23 to 82 mg Cd kg^{-1}, and 2.0 to 15.6 mg Pb kg^{-1}), with M1 soil reaching the highest Zn and Pb concentrations. The two soil samples collected from mine borders (B1 and B2) displayed moderate Zn and Cd contents (111 to 207 mg Zn kg^{-1} and 6.1 to 9 mg Cd kg^{-1}). Unexpectedly, B2 soil had 3.7 g Pb kg^{-1}, which is close to what was measured in mine soils and an order of magnitude higher than in the other mine border soil samples (170 mg Pb kg^{-1}). Finally, the two nonmining sites (NM1 and NM2) showed, as expected, low metal levels corresponding to unpolluted soils (14 to 15 mg Zn kg^{-1}, 1.3 mg Cd kg^{-1}, and 46 to 55 mg Pb kg^{-1}). All soils had a neutral to moderately alkaline pH (7.4 to 8.0). The organic C and the total N contents were higher in the two mine-border soils than those in the corresponding mine soils (M1 and M2) (2.7- to 4.2-fold differences). M3 and M4 mine soils contained more organic C than the two other mine soils (1.7- to 4.4-fold higher).

Rhizobial isolation. The number of nodulated *Anthyllis* plants used for trapping varied between the soils collected on the 8 sites. Each of the plants used for trapping was highly nodulated (>80% of plants nodulated), except for M1 and M2, with 52% and 17% of nodulated plants, respectively (see Table S3). A total of 137 isolates was considered in this study (see Table S1). One hundred twelve were newly isolated rhizobial strains from mine ($n = 40$ isolates), mine-border ($n = 34$), and nonmining ($n = 38$) sites. These 112 new strains were combined with the 25 previously isolated strains corresponding to 12 *M. metallidurans* isolates from M1 (35), 9 *Aminobacter* spp. (from M3 and M4) (39), and 4 *Mesorhizobium* spp. from M3 (39) and NM2 sites (40).

Metal tolerance of strains. All 137 *Anthyllis* isolates were screened on growth media containing increasing metal concentrations to determine Zn and Cd MIC values (Fig. 1; see also Table S1). Rhizobial isolates from all mine soils had significantly higher MIC values for Zn (MIC, 1 to 20 mM) and Cd (MIC, 0.1 to 10 mM) compared to those of isolates from the two nonmining soils (MICs, 0.02 to 0.8 mM for Zn and 0.02 to 0.5 mM for Cd; $P \leq 0.0002$) (Fig. 1; see also Table S4). Rhizobial isolates recovered from M3 samples contained the most Cd-tolerant isolates (MIC, 10 mM) (Fig. 1B). The nine isolates corresponding to the *Aminobacter* strains from M3 and M4 samples had intermediate levels of metal tolerance (see Table S1). Regarding isolates from mine-border soils, the Zn and Cd MIC values of all of the isolates from B1 were similar to those of isolates from nonmining soils (nonsignificant MIC differences; see Table S4) (Fig. 1B). Furthermore, only isolates from B2 samples displayed a mixture of both sensitive and tolerant phenotypes based on their growth on metal-containing media: (i) the first group of five isolates (STM3880, -3881, -3887, -3888 and -3976) had Zn and Cd MIC values similar to those of isolates from mine soils, and (ii) the second group of seven isolates (STM3971, -3972, -3973, -3974, -3891, -3988, and -3991) had Zn and Cd MIC values similar to those of isolates from nonmining soils (Fig. 1; see also Table S1).

Phylogenetic relationships of *Anthyllis* rhizobia using taxonomic marker genes. The majority (128 isolates) of the 137 symbiotic strains of *A. vulneraria* belonged to the genus *Mesorhizobium* and the remainder (9 isolates) belonged to the genus *Aminobacter* (see Fig. S1A).

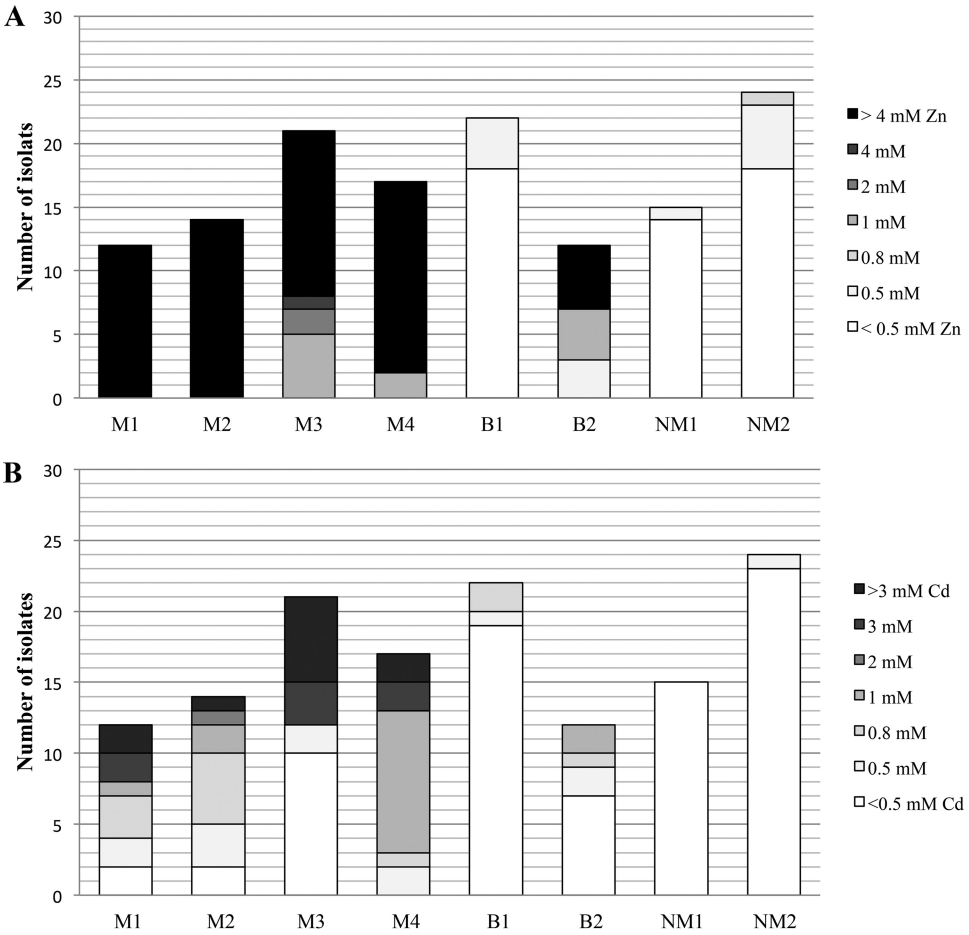

FIG 1 Zn (A) and Cd (B) MIC data of the 137 *Anthyllis* rhizobia studied and classified by their sites of origin. The OD_{600} was assessed after 2 weeks of growth in YEM medium. The results are the means of three replicates.

As a large number of isolates harbored identical 16S rRNA gene sequences, we expanded the analysis to two housekeeping genes (*recA* and *atpD*) to improve the identification of mesorhizobia and the reliability of the 16S rRNA gene phylogeny. The phylogenetic tree built from the concatenation of 16S rRNA, *recA*, and *atpD* partial sequences segregated the 137 strains studied into four clusters (TI to TIV) (Fig. 2). Clusters TI, TII, and TIII encompassed all 128 *Anthyllis* strains belonging to the genus *Mesorhizobium*, while cluster TIV contained strains from the genus *Aminobacter*. Intriguingly, these clusters successfully separated metal-sensitive from metal-tolerant strains. Clusters TI and TIII included all of the sensitive strains from the mine-border site B1, nonmining sites (TI), and the mine-border site B2 (TIII). These strains were closely related to *M. muleiense*/*M. temperatum* or *M. caraganae*. In contrast, cluster TII contained all of the intermediately tolerant and tolerant *Mesorhizobium* strains from mine sites and the five metal-tolerant strains from the mine-border site B2 and belonged to the *M. metallidurans* clade.

Identification and geographic distribution of strains belonging to *M. metallidurans*. To resolve the intraspecies-level phylogenetic structure of the *M. metallidurans* strains, reference strains of previously validated *M. metallidurans* (35) were included in all the trees (Fig. 2; see also Fig. S1). Both reference strains and strains from the current study clustered together in a site-specific manner (TII in Fig. 2) and the M3 mesorhizobial strains were phylogenetically distinct from the *M. metallidurans* clade (first branches of TII in Fig. 2). To determine whether *Mesorhizobium* strains originating from the M3 mine could be assigned taxonomically within the species *M. metallidurans*, we measured the average nucleotide identity (ANI) and estimated their DNA-DNA hybrid-

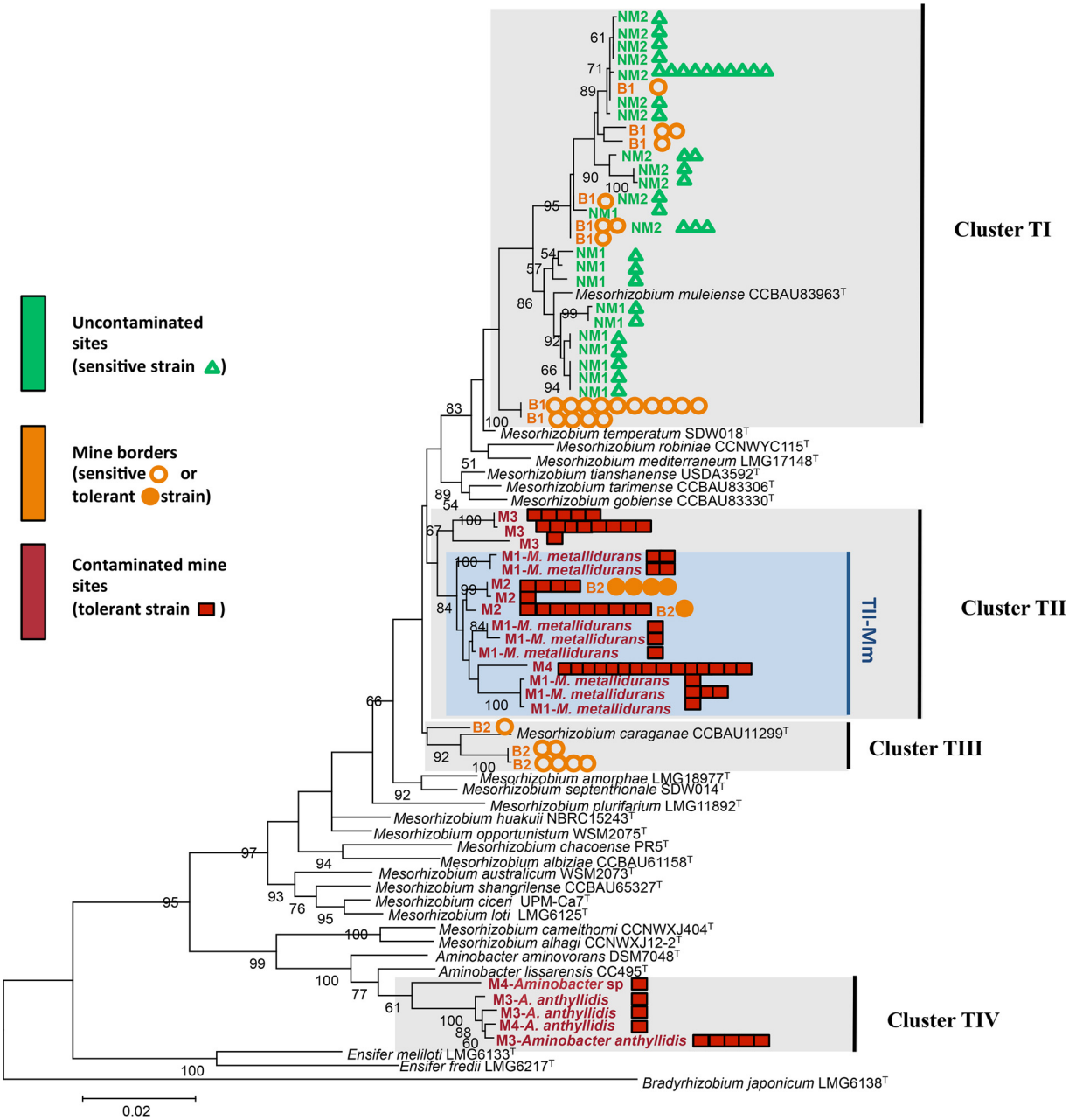

FIG 2 Maximum-likelihood concatenated phylogenetic tree based on 16S rRNA (1165 nt), *atpD* (381 nt), and *recA* (379 nt) sequences showing the relationships between the *A. vulneraria* isolates used in this study and reference strains. GenBank accession numbers are given in Table S2 in the supplemental material. Bootstrap values >50% are shown. The scale bar represents the number of nucleotide substitutions per 100 nucleotides. Individual *A. vulneraria* nodule isolates are shown using symbols colored according to site origins and tolerance (filled, MIC ≥1 mM Zn) or sensitivity (empty, MIC <1 mM Zn) to metals.

ization (DDH) values using the recently released genome sequences from strains STM4661 (M3) and STM2683^T (M1) (37). Based on current thresholds set to an ANI of >95% and a DDH of >70% for intraspecies genome-based comparisons, both the ANI (92.2%) and the DDH estimates (47.6%) showed that these strains did not belong to the same species and most probably represented 2 distinct *Mesorhizobium* species. Therefore, only the branches in the subcluster labeled TII-Mm (in dark gray in Fig. 2) were assigned to strains of *M. metallidurans*. *M. metallidurans* strains were all Zn-tolerant (MIC, ≥4 mM), and they represented 46% of the 137 isolated rhizobial *Anthyllis* strains, 67% of the 69 metal-tolerant strains, or 77% of the tolerant mesorhizobia. They were

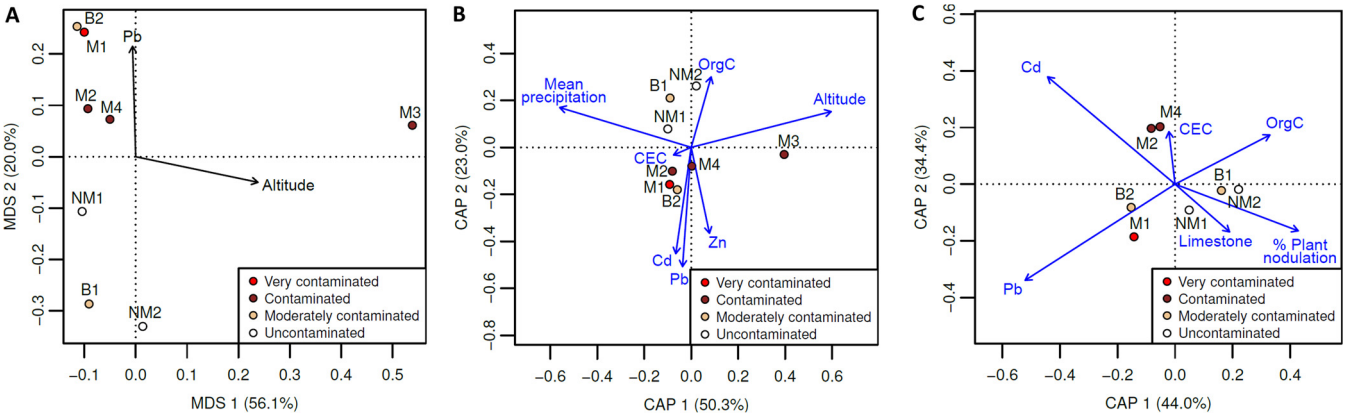

FIG 3 Ordination analysis of the rhizobial communities. The weighted UniFrac distance matrix from the OTU table that was clustered at a threshold of 100% was used as the input matrix for generating the ordination graph. The points are color-coded according to the degree to which the sites were contaminated with heavy metal compounds. (A) Unconstrained PCoA with the environmental variables plotted as directional arrows using Envfit (only variables with $P < 0.2$ are shown). (B and C) Constrained CAPs of the rhizobial communities. Given that the community from Eylie (M3) was distantly related to the other rhizobial communities in panel A, and to further resolve the effect of heavy metals on the communities at other sites, M3 was removed prior to the constrained ordination analysis presented in panel C.

found only in mine sites, except for the M3 isolate that contained no *M. metallidurans* but another closely related metal-resistant *Mesorhizobium* genospecies.

Heavy metals in soils shape *Anthyllis* rhizobial composition. To study the effects of soil and topoclimatic factors on bacterial diversity across the sites, we calculated the weighted UniFrac matrix based on all the 16S rRNA, *recA*, and *atpD* concatenated sequences from the 137 *Anthyllis* strains. Data obtained from the principal-coordinate analysis (PCoA) showed that about 76% of the variation could be explained by axes 1 and 2 (Fig. 3A). The rhizobial community structure of M3 was vastly different from the communities of the other sites along the first principal coordinate, while the second coordinate showed that the rhizobial community diverged between the contaminated and uncontaminated sites. Envfit analysis showed that elevation was the first factor explaining the divergence of site M3; M3 was located at a higher elevation (960 m) than the other sites (164 to 703 m). Furthermore, axis 2 of the PCoA ordination space was most strongly explained by soil Pb content. Constrained analysis of principal coordinates showed again the importance of elevation in distinguishing M3 from the other communities, while soil organic C content drove all but one of the nonmine and mine-border communities, and Cd, Pb, and Zn drove the rhizobial community composition of the contaminated sites and site B2 (Fig. 3B). To better resolve the effects of the different heavy metals on the rhizobial communities, the highly divergent M3 community was removed and the constrained analysis of the *Mesorhizobium*-dominated communities was repeated. This new analysis further indicated that the rhizobial communities at the M2 and M4 mining sites were most highly influenced by the soil Cd concentrations, while the M1 mine community was more strongly driven by soil Pb concentrations (Fig. 3C). Rhizobia originating from mine-border site B2 were also more highly driven by Pb concentrations unlike at the corresponding mine (M2). Last but not least, the proportion of nodulated plants increased with decreasing heavy metal contamination at the respective sites.

Occurrence and phylogeny of the *cadA* gene in the *Anthyllis* rhizobial collection. A fragment of the *cadA* gene, which encodes a metal efflux protein, was amplified in each of the mesorhizobial isolates from mine soils (M1 to M4) and in isolates from the mine-border B2 that displayed high metal tolerance (see Table S1). Additionally, the *cadA* gene was detected in all nine of the intermediately metal-tolerant *Aminobacter* strains from M3 and M4. In contrast, the *cadA* fragment was not amplified from isolates originating from the nonmining soils or from the metal-sensitive isolates in the mine-border soils (B1 and B2). Thus, the *cadA* marker was detected in each of the isolates from 4 sites, was not detected in isolates from 3 sites, and was detected in some of the

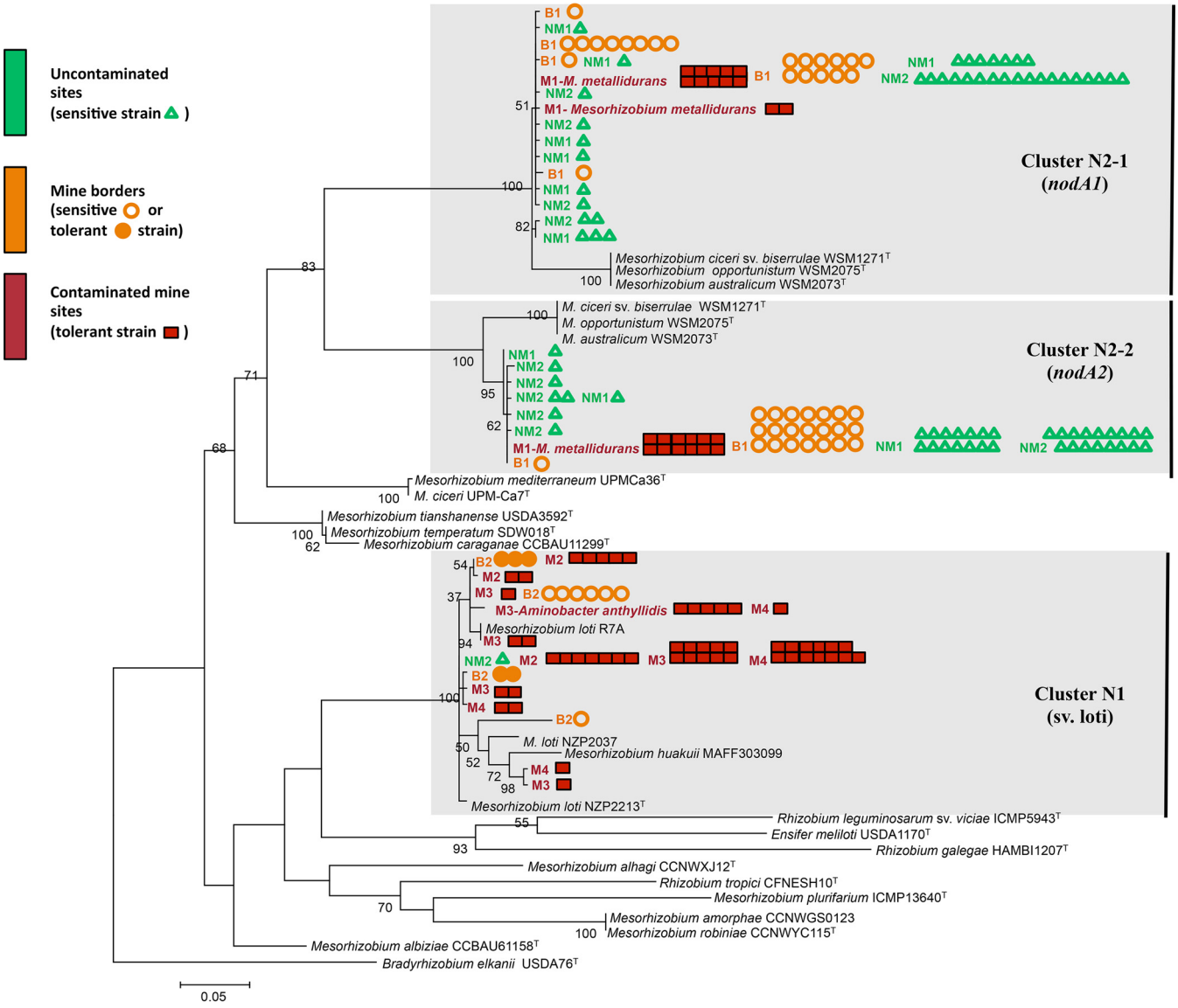

FIG 4 Maximum-likelihood phylogenetic tree based on partial *nodA* gene sequences (394 nt) showing the relationships between the *A. vulneraria* isolates obtained in this study and reference strains. GenBank accession numbers are given in Table S2 or next to the reference strains. Bootstrap values >50% are shown. The scale bar represents the number of nucleotide substitutions per 100 nucleotides. Individual *A. vulneraria* nodule isolates are shown using symbols colored according to site origins and tolerance (filled, MIC ≥1 mM Zn) or sensitivity (empty, MIC <1 mM Zn) to metals.

isolates from one site (B2). The partial CadA protein sequences of each of these metal-tolerant mesorhizobia were closely related to the CadA sequence of *M. metallidurans* STM2683^T (82 to 100%; see Fig. S2). These mesorhizobial strains belonged to either *M. metallidurans* or the new metal-tolerant genospecies (see cluster TII in Fig. 2). The more distantly related *Aminobacter* isolates harbored CadA genes that were phylogenetically distinct from those of mesorhizobial strains (see CIII in Fig. S2).

Sequence analysis of the nodulation gene *nodA*. Isolates from M1, B1, NM1, and NM2 (except one strain) possessed two copies of the nodulation gene *nodA* (*nodA1* and *nodA2*) (Fig. 4; see also Table S1). These two copies shared high identities with those of *M. metallidurans* STM2683^T (98.7 to 100%) and formed sister clades with the respective *nodA1* and *nodA2* genes from *M. ciceri* symbiovar (sv.) *biserrulae*, *M. opportunistum* and *M. australicum* isolated from *Biserrula pelecinus* L. (Fig. 4). In contrast, the unique copies of the *nod*A gene from M2, M3, M4, and B2 isolates, and from one NM2 isolate, were related to those of the *Mesorhizobium* strains isolated from *Lotus* sp. and were categorized with the symbiovar *loti* (cluster N1 in Fig. 4; see also Table S1).

DISCUSSION

Rhizobia associated with metal-tolerant *Anthyllis* plants are important ecological players responsible for the entry of biologically fixed nitrogen into metal-contaminated soils. By promoting *Anthyllis* growth, they facilitate the activation of post-mining site restoration. We show a strong impact of heavy metal contamination on *Anthyllis*-associated rhizobial communities with respect to metal tolerance properties and taxonomic species composition, but not with respect to symbiotic *nodA* diversity.

Isolates from mine sites contained a high proportion of Zn- and Cd-tolerant rhizobia compared with the isolates from nonmining sites. This result is in agreement with previous studies that found a positive correlation between metal tolerance and levels of metals in the environment (15, 22, 23). The results of our study also show that this positive correlation between rhizobial metal tolerance and soil metal concentrations is more evident at the community scale rather than at the cellular scale. In this regard, at the intermediately polluted mine-border site B2, rhizobial isolates exhibited an all-or-nothing phenotype, i.e., either tolerant or sensitive to soil metal pollution, while at the community level, we observed an intermediate phenotype comprising a mixture of tolerant and sensitive individuals. Mine-border soils were much less contaminated compared to mine soils and contained higher levels of organic matter that might decrease metal mobility and, in turn, metal availability in soils (41, 42). This might result in a more heterogeneous soil environment with hotspots of metal-available niches interspersed among niches with less metal availability at the microscale. The presence of metal-tolerant isolates at the B2 site might be linked to the high levels of Pb contained in the soil in this site, which were similar to levels of mine soils. Zn tolerance is often associated with Cd and/or Pb tolerance. For example, the P_{IB}-type ATPase ZntA or CadA efflux transporters can confer resistance to Zn, Cd, and Pb (43). Therefore, the higher number of metal-tolerant phenotypes we found in the B2 site may result from the selection pressure exerted by Pb in the soil.

The higher number of metal-tolerant phenotypes in metal-contaminated soils might be related to the acquisition of metal-resistance mechanisms, such as metal-efflux transporters (12). The presence of *cadA*, previously identified as contributing to high Zn resistance in *M. metallidurans* STM2683^T (38), coincides with the metal-tolerance phenotype in rhizobia from our mine soils and the mine-border soil B2. With 100% of *cadA* detection in the metal-tolerant isolates and no detection in any of the mesorhizobial sensitive isolates, *cadA* appears to be a good molecular marker of metal-tolerant *Anthyllis* symbionts. Although different mechanisms might be involved in Zn-Cd-Pb tolerance, the omnipresence of *cad*A among these symbionts suggests that different tolerant populations, even the divergent Eylie mine M3 rhizobial community, may share common molecular determinant(s) and genomic islands for coping with heavy metal-contaminated soils. Similarly, a homogenous genetic system restricted to a small part of the genome was recently shown to be shared among closely related *Acmispon* mesorhizobia in nickel-contaminated soils (44).

In addition to metal tolerance, these analyses showed that *A. vulneraria* is nodulated by a large variety of species, including putative new species of *Mesorhizobium* and *Aminobacter*. However, *A. vulneraria* was preferentially nodulated in soils by strains belonging to the genus *Mesorhizobium* (93% of isolates), which has been historically associated with *Anthyllis* (45, 46). A new metal-tolerant genospecies was also found in the M3 site, revealing species variability not only between contaminated and uncontaminated soils, but also within similar metal-polluted sites.

Multivariate analyses based on the weighted UniFrac method confirmed that the rhizobial community recovered from the M3 mine was highly divergent from the communities at the other sites. Given the limited number of environmental variables collected for this study, the altitudinal location of M3 relative to the other sites was the only variable that most strongly differentiated site M3. Since altitude in itself does not drive soil bacterial communities (47) but rather drives other climatic variables that do influence biotic processes (48), it is highly likely that the mean annual precipitation, which was negatively

correlated with altitude, impacts the rhizobial community at site M3. Another possibility is that the M3 mine, located in the Pyrénées-Orientales state of southwestern France, experiences additional topoclimatic conditions that are different from the other mine regions and/or different regional and endemic vegetation that selects for divergent microbial communities (26, 28). Moreover, exchanges of metal ore and/or mining company staff between M1/M2 and M4 sites (J. Escarré, personal communication) might explain some kinship between soil substrate and associated bacterial communities.

Among the sites other than M3, the constrained analysis showed that bacterial communities were driven by soil Pb content at the M1 and B2 sites and by Cd concentrations at the M2 and M4 mines, and that, generally, heavy-metal contamination in the soil reduced the efficiency at which plants were nodulated. Also, as mentioned earlier in our discussion, despite site B2 being a mine-border site, the concentration of Pb in the soil at this site was equivalent to those of the other contaminated sites, while Zn and Cd concentrations were closer to what was found at other mine-border and nonmining sites. This likely explains the strong effect of soil Pb on the rhizobial community structure observed at site B2. In the absence of pollutants, nonmining sites also display distinct mesorhizobial lineages that, although not significant here, might be impacted by nutrient-based parameters, notably soil organic carbon and nitrogen contents. This may influence the mesorhizobial diversity distribution, as found for mesorhizobia from legumes grown in barren or rich soil fields (49). The results of our study demonstrated that heavy metal levels strongly affected rhizobial diversity by selecting different genospecies, in contrast to results of previous studies that showed no effect of heavy metals on the rhizobial species diversity (23, 50). Given that those studies were focused on *Rhizobium leguminosarum* and *Ensifer medicae* associated with *Trifolium* or medics, respectively, this discrepancy may be attributed to the fact that these two legumes trigger symbiosis with a limited number of rhizobial species, which contrasts with *Anthyllis*, which appears to be promiscuous with respect to nodulation. Similarly, a species shift toward a copper-tolerant rhizobial lineage on the promiscuous plant *Phaseolus*, when grown in copper-treated soils, was also reported by Laguerre et al. (51). These studies provide evidence of the usefulness of such legumes for obtaining higher species diversity among rhizobia that can adapt to particular soil constraints.

When using the *nodA* marker, the geographic distribution of isolates in the current study differed from that obtained using the 16S rRNA or housekeeping genes. Such a discrepancy was not surprising given that the *nodA* gene phylogenies tend to resolve symbiotic host associations, while the 16S rRNA and housekeeping genes better reflect the taxonomic position of rhizobia. Isolates from the Cévennes area (M1, B1, NM1, and NM2 sites) generally harbored two complete copies of *nodA* that were phylogenetically related to those of *M. metallidurans* STM2683^T, an efficient symbiont of *Anthyllis* (39). Each of the isolates from the four remaining sites (M2, M3, M4, and B2) harbored *nodA* genes belonging to a different lineage and that corresponded to the symbiovar *loti*, suggesting a cross-nodulation activity between *Lotus* and *Anthyllis*. The presence of two *nodA* lineages among the isolates nodulating *Anthyllis* may reflect a different host spectrum or a different symbiotic behavior with *Anthyllis* or *Lotus* legumes; these could be two symbiovars within *M. metallidurans* (the symbiovar *loti* and another related to *Anthyllis*). The prevalence of this *nodA* pattern might be related to the presence of the compatible host plant (*A. vulneraria* and/or *Lotus corniculatus*) in their proximate environments, as the legume host can influence the spatial distribution of isolates according to its own geographic distribution (52). Indeed, *L. corniculatus* was commonly found at the Saint-Bresson (M2 and B2) and M3 sites but not at the M1 mine. The phylogenies presented in this study strongly suggest that *Lotus* symbionts from the Saint-Bresson, M3, and M4 sites transferred *nod* genes by horizontal transfer to *Mesorhizobium* isolates nodulating *Anthyllis* spp. at these three sites. Interestingly, the first evidence of such horizontal gene transfer was found among *L. corniculatus* mesorhizobia (53).

In conclusion, our results demonstrate that the intrinsic properties of each site influence the composition of rhizobial communities based on their metal tolerance and genospecies but not according to their symbiotic *nodA* characteristics. Thus, *Anthyllis*

mesorhizobia exhibit related but distinct species or genospecies distributed across different sites, thereby resulting in distinct sets of species at respective sites. Mesorhizobia show a high variability and capacity to adapt to local and extreme edaphic conditions that are site specific. This agrees with data revealing taxon-area biogeography within mesorhizobia symbiotic to, for instance, chickpea (54) and liquorice (55) legumes, as well as extensive species diversification with the root-nodulating members of the genus *Mesorhizobium*. Therefore, to remediate metal contamination, this work suggests that native plants and microorganisms that are better adapted to local pedoclimatic conditions should be used in phytostabilization strategies based on *Mesorhizobium*-legume interactions.

MATERIALS AND METHODS

Site and soil sampling. We sampled a total of eight sites from the Cévennes, Pyrénées Ariégeoises (France), and Rheinland (Germany) regions. Soil was sampled (0 to 10 cm) from four decommissioned mines, including the Saint-Laurent-le-Minier district in the Cévennes area, Avinières (M1; 43°55′57.39″N, 3°39′58.5″E, elevation of 164 m, mining operations from 1872 to 1914), Saint-Bresson (M2; 43°57′24.57″N, 3°39′25.69″E, elevation of 425 m, mining operations closed in the early 20th century), the Eylie mine in the Pyrénées Ariégeoises (M3; 42°49′59.59″N, 0°56′15.99″E, elevation of 960 m, mining operations from 1850 to 1953), and one old mine (closed in 1883) now in the Schlangenberg nature reserve, Breinic, Germany (M4; 50°44′35.33″N, 6°15′19.21″E, elevation of 294 m). The two soil samples from mine-borders were from Avinières (B1; 43°55′53.90″N, 3°39′44.72″E, elevation of 250 m) and from Saint-Bresson (B2; 43°57′28.06″N, 3°39′27.25″E, elevation of 455 m). Finally, two samples from nonmining soils were collected in the Cévennes area, 25 kilometers southwest from Saint-Laurent-le-Minier: one at Col du Vent (NM1; 43°44′41.79″N, 3°27′38.16″E, elevation of 703 m) and one at La Prunarède (NM2; 43°52′14.14″N, 3°30′39.77″E, elevation of 707 m). All the sites of the Cévennes area were located within a 20-km radius, while M3 was about 300 km southwest and M4 was 1000 km north.

Soil analyses. For each of the eight sites, five to ten soil subsamples were collected across the site at a depth of 1 to 10 cm and were homogenized and sifted through a 2-mm mesh sieve for further chemical soil analyses ($pH_{[H2O]}$, organic carbon [C], total nitrogen [N], and cation exchange capacity [CEC]). Heavy metal concentrations were determined from 20 g of air-dried soil that were shaken for 30 min with 100 ml of AAAc-EDTA extracting solution (0.5 M ammonium acetate, 0.5 M acetic acid, 0.02 M Na_2EDTA, pH 4.65). The extracts were filtered on folded Schleicher & Schuell grade 595 1/2 filters (125-mm diameter) and analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Vista-MPX; Varian Inc.).

Rhizobial isolation. We collected seeds from the population of *A. vulneraria* (subspecies *carpatica*) naturally growing in the Avinières mine. Germinated seeds were grown in tubes containing 10 g of soil samples on top of clay granules. Six weeks later, nodules were recovered and were surface sterilized with 300 μl of 3% calcium hydrochloride for 2 min, rinsed 5 times with distilled water, and crushed in 50 μl of sterile distilled water (35). The bacterial suspensions were streaked on yeast extract mannitol (YEM) agar plates and incubated at 28°C (35). Bacterial colonies appeared after 2 to 4 weeks of incubation and single colonies were checked for purity by repeated streaking on YEM agar. Isolates were stored at −80°C in 20% glycerol (vol/vol). In this study, previously isolated rhizobia from soils of Avinières, Eylie, and Breinic mines were included (35, 39). The list of *Anthyllis* rhizobia used in the present study is presented in Table S1 in the supplemental material.

DNA extraction and sequencing. Genomic DNA was extracted from the isolates according to a standard phenol-chloroform extraction method (56) and stored at −20°C. Near full-length 16S rRNA genes were amplified by PCR as previously reported (35). Internal fragments of approximately 500 bp of *recA* and *atpD* were amplified using primer pairs *recA* 63f/504r and *atpD* 294f/771r (57) with an annealing step of 30 s at 57°C. For *nodA*, 500-bp fragments were amplified with *nodA* F21Meso/R591Meso primers (58) using an annealing step of 30 s at 55°C. Some isolates were characterized by the presence of two copies of the *nodA* gene. In that case, specific primers were designed on the basis of the type strain of *M. metallidurans* STM2683^T draft genome (37) and used to amplify a 700-bp product. To amplify the *nodA1* gene, which corresponds to the copy of *nodA* inside the *nodABC* operon, we used primers STM3315 forward (5′-TCTGCTCGACGCGACAAGC-3′) and STM3316 reverse (5′-TCTTCGGTGCCGTCAGCG-3′). To amplify *nodA2* (corresponding to the copy of *nodA* in the *nodAFEG* cluster), primers STM3313 forward (5′-TGCCTCTATGCCGCGATGAGC-3′) and STM3314 reverse (5′-ATGAGTCCGGCTGGTCTGCG-3′) were used for PCR using the following cycling procedure: 35 cycles of denaturation at 96°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 45 s. A 700-bp fragment of the *cadA* metal-resistance gene encoding a P_{1B}-type ATPase efflux transporter (HE820903) was amplified between the metal-binding site from the TM6 motif (CPC) and the ATP-binding domain from the H8 motif (GDGXNDXP) using primers designed from the STM2683^T genome: STM2612 forward (5′-GACCGAGCGGTTCATCG-3′) and STM2613 reverse (5′-CGGTCAGCATCACAGCAG-3′) (38) with an annealing step of 45 s at 57°C. All PCR amplifications were performed with the GO *Taq* polymerase (Promega, Madison, WI, USA). PCR products were run on 1% agarose gels stained with ethidium bromide in 1× Tris-acetate-EDTA (TAE). The corresponding bands were gel extracted, purified with a Qiagen kit, and then sequenced by GenoScreen Company (Lille, France) using a BigDye Terminator v3.1 kit.

Estimation of bacterial tolerance to metals. MICs were determined in YEM liquid medium. MIC was defined as the lowest metal concentration that inhibited visible growth. One hundred fifty microliters of

medium containing increasing concentrations of metals (0.02, 0.05, 0.1, 0.3, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 18, 20, 24, and 32 mM $ZnSO_4$ and 0.02, 0.05, 0.1, 0.3, 0.5, 0.8, 1, 1.5, 2, 3, 5, 7, 10, and 12 mM $CdCl_2$) or no metal (positive control) were inoculated with 10 μl of the rhizobial strains that were precultured for 4 days and adjusted at an optical density at 600 nm (OD_{600}) of 0.5 prior to inoculation. The cultures were incubated at 28°C with orbital shaking (250 rpm) and growth was monitored for 2 weeks by reading the OD_{600} using a spectrofluorometer (TECAN Infinite M200). MIC assays were performed in triplicate for each isolate. The nonparametric Kruskal-Wallis test was used to assess the significance of MIC differences between paired groups with *P*-value adjustment for multiple comparisons by the false discovery rate (FDR) method using R statistical software.

Phylogenetic and multivariate analyses. Phylogenetic analyses of 16S rRNA, *recA*, *atpD*, and *nodA* were performed using nucleotide sequences, and those of *cadA* were performed using amino acid sequences due to high nucleotide divergences within ATPase pumps. Sequence alignments were performed with MEGA 6.06 v6140226 (59) and manually optimized with GeneDoc v2.7.000 (60). Phylogenetic trees were inferred by the neighbor-joining method using Kimura two-parameter distances or by the maximum likelihood (ML) method using General Time Reversible (GTR) in the MEGA software. Confidence of the tree branches was estimated with 1000 bootstrap repetitions. Trees were rooted using *Bradyrhizobium japonicum* sequences for 16S rRNA, *recA*, and *atpD* phylogenies, *B. elkanii* for *nodA* phylogeny, and *Chelativorans multitrophicus* for CadA phylogeny as outgroups. Additionally, an ML tree was constructed based on concatenated sequences obtained from 16S rRNA, *atpD*, and *recA* fragment alignments as mentioned above. The concatenated sequences were then clustered at 100% similarity threshold in QIIME v.1.7.0 (61). QIIME was also used to build a reference tree of the representative sequences from each operational taxonomic unit (OTU) using the FastTree method and a weighted UniFrac matrix (62) was generated. Unlike the unweighted UniFrac, which takes into consideration only the community membership, the weighted UniFrac metric calculates the beta diversity among communities using membership and the relative abundance of the respective OTUs, i.e., their community structure. The Vegan package (63) in the R statistical package (http://www.r-project.org/) was used to draw the unconstrained principal-coordinate analysis (PCoA) plot of all the rhizobial communities. The Vegan function Envfit was used to calculate the effect of the environmental variables on the rhizobial communities. Given the low numbers of sites, a high statistical significance could not be obtained using Envfit, so only those variables with a $P < 0.2$ were reported in the study, with the highest *P* value of 0.165 assigned to soil Pb content. Before and after removing the outlier site (Eylie, M3) from the data set, a stepwise model evaluation on the UniFrac matrix was done to determine the best combination of environmental predictors that could explain the rhizobial community composition. The model with the lowest Akaike information criterion (AIC) value (-589.04 and -526.96 for the model with and without M3, respectively) contained the predictors of altitude, mean precipitation, Pb, Zn, CEC, Cd, and organic C for the model including site M3, and Pb, Cd, CEC, organic C, percent plant nodulation, and limestone content for the model excluding site M3 (refer to Table S3 for further details on each variable). These higher predictor models were plotted in a constrained analysis of principal coordinates (CAP). For both the Envfit and stepwise model calculations, the following environmental variables were included: plant nodulation percentage, limestone, clay, sand, Zn, Pb, Cd, organic C, and total nitrogen contents, CEC, soil pH, altitude, and mean annual precipitation (see Table S3). Mean annual temperature was excluded because of its correlation with mean annual precipitation (Spearman's rank correlation of 1). To determine the levels of correlation of the environmental predictors, Spearman's rank correlation analysis was done across all of the available predictors using the R package Hmisc. The resulting correlation results were *P*-value adjusted using the false discovery rate method and were visualized as a network in Gephi v0.8.2 using the Force Atlas2 algorithm (see Fig. S3).

ANI and DDH. To estimate whether the two metal-tolerant *Mezorhizobium* strains, *M. metallidurans* STM2683^T and *Mesorhizobium* sp. STM4661 isolated from the M1 and M3 mines, respectively, and for which the genomes have recently been released (37), belong to separate species, we calculated the average nucleotide identity (ANI) (64, 65) and *in silico*-based DNA-DNA hybridization (DDH) estimates (66). The alignment options used for the ANI calculation were 70% minimal identity over more than 700 bp in fragment windows of 1000 bp with a 200-bp step size on reciprocal best hits (two-way ANI). For the DDH measurement, we used the genome-to-genome distance calculator (GGDC) with the BLAST+ alignment method and the recommended formula (identities/high-scoring segment pair lengths). The probability that DDH is >70% was estimated as described by Meier-Kolthoff and colleagues (66).

Accession number(s). The nucleotide sequences obtained in this study were deposited in the GenBank database under the following accession numbers: KP749909 to KP749919 for the 16S rRNA gene, KP150565, KP150567 to KP150570, and KP942491 to KP942514 for the *recA* gene, KP942467 to KP942490 for the *atpD* gene, KP729628 to KP729637 and KP735732 to KP735751 for the *nodA1* gene, KP735752 to KP735771 for the *nodA2* gene, and KP687433 to KP687449 for the *cadA* gene (see Table S2 for all accession numbers of isolates and reference strains in the supplemental material).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01735-16.

TEXT S1, PDF file, 1.1 MB.

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